Docket No.: HO-P02102US2
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Suzanne Fuqua, et al.

Application No.: 10/052,092

Group Art Unit: 1634

Filed: January 18, 2002

Examiner: Switzer, J.C.

For: METHODS AND COMPOSITIONS IN BREAST CANCER DIAGNOSIS AND THERAPEUTICS

DECLARATION UNDER 37 CFR §1.132

Dear Sir:

I, Suzanne A. Fuqua, Ph.D., do hereby depose and say as follows:

1. I am a United States citizen residing at 5410 Drakeview Court, Sugar Land, TX, 77479, USA.

2. I am an employee of the assignee of the above-referenced patent application, I am an inventor of said application, and I have read the contents of said application.

3. I am a Professor in the Breast Center at Baylor College of Medicine in Houston, Texas. I am skilled in the area of molecular genetics and disease, particularly breast cancer. A resume describing my experience is attached to this declaration.

The Examiner is alleging in the outstanding Office Action that the presently pending claims are rejected under 35 U.S.C. §112, first paragraph as not being enabled by failing to teach how to make and use the invention commensurate with the scope of the claims. This is an inaccurate assessment of the nature of the present invention and the technology involved for this particular application, as I will address herein and in the attached Powerpoint Presentation having 14 slides.

Specifically, the Examiner is alleging that the specification does not provide enablement for methods of diagnosing breast cancer or methods of classifying breast cancer in an individual. This is erroneous, given that the specification in paragraphs **[0330]** and **[0331]** (which is also the first slide attached herewith) illustrate that the K303R mutation (which resulted from the A908G mutation in the nucleic acid sequence) is associated with breast cancer. In particular, in patients having invasive breast tumors, the cancer is found in

62% of patients (33 out of 53 individuals), regardless of the status of spread to the axillary lymph nodes.

We demonstrated that there is a proliferative advantage for cells expressing the A908G mutation due to their hypersensitivity to the hormone estrogen, as illustrated in the second slide. On the left of the slide is a transactivation assay of human MDA-MB-231 breast cancer cells expressing either the wild type (WT) or mutant in the presence of increasing levels of estrogen. This assay determines the activity of the two receptors in cells. Greater transcriptional activity occurs at lower levels of estrogen with the mutant, compared to the WT receptor. Quantitatively, the mutant receptor exhibits approximately a two hundred fold increase in estrogen sensitivity. These differences are most apparent at the low concentrations of hormone, equivalent to the concentrations of hormone estimated to be in postmenopausal women. The cell growth assays, as shown in the middle of the slide, demonstrate that even with low levels of estrogen there is enhanced growth in the presence of low levels of hormone. Furthermore, the mutant is maximally stimulated to grow even at low levels of hormone. This difference in hormone stimulation provides the mutant cells with a distinct proliferative advantage.

This would have predictive implications in a postmenopausal patient. That is, in postmenopausal women there are normally reduced levels of estrogen, with subsequent reduced receptor activity and decline in breast cell growth. Thus, as women age, the stimulation of breast cell growth due to the influence of the WT receptor diminishes. However, these experiments demonstrate that cells expressing the mutant would continue to grow well and be stimulated by the estrogen hormone, even at lower levels of the hormone.

On the right side of the second slide is a standard *in vitro* modified Boyden chamber invasion assay well-accepted by those of skill in the art. This assay characterizes the ability of the WT *vs.* mutant cells to chew up a surrounding environment comprised of extracellular matrix, and to migrate through a barrier membrane. Therefore, it is an accepted test for predicting invasiveness of particular cells such as those comprising the A908G mutation. The cells used in this experiment were MCF-7 human breast cancer cells which normally express WT receptor. But similar results were obtained in all cells examined with the invasion assay. The cells comprising the mutation had a significantly higher invasion index than WT cells. Particularly at reduced levels of hormone, where the estrogen has been

depeleted experimentally, the cells expressing the mutant receptor are greater than 15 times the amount of invasion as vector-transfected, control cells.

In the next slide, we demonstrate the presence of the nucleic acid sequence mutation in 90% of tested primary invasive breast cancers from postmenopausal women, wherein the cancers were ERα-positive, node-negative, and greater than 2 cm in size. Biopsies were taken at time 0 before surgery, followed by biopsies two weeks or twelve weeks during antiestrogen (tamoxifen) therapy administration, and the presence of cells having the mutation is monitored. In 90% of women with primary invasive breast cancers, the mutation was identified. Furthermore, as illustrated on the bottom right, over time cells having the wild type "A" nucleotide in the ER sequence at 908, were being selected against, resulting in cells with the "G" 908 mutation to predominate in the remaining cells of the residual tumor after therapy. This result shows that the mutation is frequent in postmenopausal women with ER-positive disease, and that the mutation may be resistant to therapies such as tamoxifen.

We also addressed the finding that only A908G mutations are identified in tumors, as opposed to the nucleotide changes, A908T or A908C tumors, as demonstrated in the fourth slide. The other two possible nucleic acid alterations were generated *in vitro*, and subjected to a transactivation assay as before. The transactivation assay illustrated therein suggests that the A908G mutation is the only mutation at position 908 that has a statistically sensitive change in fold induction at the lowest levels of estrogen, confirming that only the A908G mutation, and not the others, render the cell hypersensitive to estrogen. This explains why a cell comprising this mutation is being selected for over time as a patient is being treated and the patient thus progresses of her disease.

It is known in the art that some proteins that help control growth of a breast cell are regulated by the ER-α receptor, so a protein-protein binding assay was performed to characterize any differences between the binding of known ER co-activators, as illustrated in slide 5, and WT or the mutant receptor. With even minute amounts of estrogen, the co-activators TIF-2 and AIB1 bind together with the mutant receptor, in contrast to the WT receptor. Much more hormone is needed before WT and co-activator binding occurs. These complexes then bind to DNA dynamically, particular at specific promoter sites, such as the exemplary pS2 promoter, and this is assayed in slide 6 of the attachment. The ER-coactivator complexes comprising the mutant receptor remain bound on the DNA, as opposed to

modulating between being on the DNA and then off the DNA, as with the WT receptor. In addition, necessary ER co-regulatory proteins, such as p300 are only in a short-lived complex with WT ER, but continue to be bound to the mutant receptor for long periods. Thus, the mutant receptor is complexing abnormally with the co-activators, retaining them in a transcriptional complex, and continuing to stimulate growth of tumors by sustained transcription and expression of particular downstream genes involved in the growth of the tumor cell.

Conversely, mutant ER-α activity is resistant to known ER regulatory proteins, called co-repressors, that normally inhibit its activity, such as MTA2, NcoR, and BRCA1 (see slides 7 and 8). These corepressor proteins are the normal "brake" for the ER hormone response system. For two different human breast cancer cell types harboring the mutation, transactivation assays similar to those described above demonstrate that fold induction does not change significantly in the presence of added MTA2, although it decreases significantly with the WT receptor. Thus, the mutant is resistant to the transcriptional inhibitory effects of MTA2, NCorR, and the breast cancer tumor suppressor gene, BRCA1.

Therefore, the mutant ER-α receptor is being activated continually to facilitate downstream gene expression and, furthermore, is not being inactivated. To explain such activities, we performed biochemical studies for characterization of the mutant. We noted that the WT ER-α comprised an acetylation motif RSKK, similar to the consensus acetylation RXKK motif found in proteins such as the tumor suppressor gene, p53 and the transcription factor GATA-1. This motif is mutated in the A908G mutant, which produces an RSKR motif. As demonstrated by in vitro acetylation assay, shown on the right of slide 9, the mutant is not acetylated. We then performed cellular total kinase assays using radiolabeled phosphorus in MCF7 human breast cancer cells to determine if the absence of acetylation affected potential phosphorylation, and we determined that the mutant are highly phosphorylated, compared to WT (slide 10).

To identify potential kinases that affect mutant ER-α phosphorylation, we reviewed the primary sequence and identified a site in the WT ER-α, $KKNS_{305}$, that is not present in the mutant ER-α, $KRNS_{305}$. For both PKA and PAK1 (as well as Akt—not shown), the mutant ER-α showed considerably more phosphorylation (slide 11).

Finally, we further demonstrated that phosphorylation and acetylation are coupled by producing our own mutant S305D (two residues from the site affected by A908G mutation) that results in a nucleotide change which mimics phosphorylation at this serine, and showed that mimicking phosphorylation at this site increases estrogen sensitivity in transactivation assays, just like what we saw only with the A908G.

Therefore, we have shown that not only does the A908G mutation associate with breast cancer, but that the molecular biological characterization of the mutant gene product explains the selective advantage the mutation confers to the cancerous cell. As such, we have shown how to make and use the invention by emphasizing the association between the A908G mutation and breast cancer and the molecular mechanism behind its hypersensitivity to estrogen and subsequent selective advantage it provides.

Furthermore, in the Office Action the Examiner notes that the specification provides examples of cases where the mutation is present in non-cancer tissues. This is an inaccurate evaluation of the data presented and the corresponding technology. We note in paragraph **[0319]** of the specification that variant A908G ERα sequence was detected along with WT sequence in the normal adjacent DNA (N Adj.) and the typical hyperplasia (TH) DNA from a patient, but that the ***normal distant tissue (N Dis.) displayed only WT ERα sequence***. It is known in the art that this is to be expected in regions of increased risk if the mutation confers a selective advantage to these cells, which, as described above, this mutation clearly does. More importantly, it is known in the art that techniques for obtaining samples *in vivo* from the breast are likely to have contaminating wild-type cells, so at least some samples may comprise cells from adjacent non-cancerous tissue lacking the A908G mutation.

In summary, as one of skill in the art, it is clear from the above data that there is an association between the A908G mutation of ER-α and the development of breast cancer, including invasive breast cancer. The data from the specification and also that provided herewith illustrate this convincing association, which provide ample support for the enabled pending claims.

4. I hereby declare that all statements made herein on my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/15/03

Suzanne Fuqua, Ph.D.